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Genome scan of *Diabrotica virgifera virgifera* for genetic variation associated with crop rotation tolerance

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Abstract: Crop rotation has been a valuable technique for control of *Diabrotica virgifera virgifera* for almost a century. However, during the last two decades, crop rotation has ceased to be effective in an expanding area of the US corn belt. This failure appears to be due to a change in the insect's oviposition behaviour, which, in all probability, has an underlying genetic basis. A preliminary genome scan using 253 amplified fragment-length polymorphism (AFLP) markers sought to identify genetic variation associated with the circumvention of crop rotation. Samples of *D. v. virgifera* from east-central Illinois, where crop rotation is ineffective, were compared with samples from Iowa at locations that the behavioural variant has yet to reach. A single AFLP marker showed signs of having been influenced by selection for the circumvention of crop rotation. However, this marker was not diagnostic. The lack of markers strongly associated with the trait may be due to an insufficient density of marker coverage throughout the genome. A weak but significant general heterogeneity was observed between the Illinois and Iowa samples at microsatellite loci and AFLP markers. This has not been detected in previous population genetic studies of *D. v. virgifera* and may indicate a reduction in gene flow between variant and wild-type beetles.

Key words: AFLP, microsatellite, selection, western corn rootworm

1 Introduction

The western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Col., Chrysomelidae) is a serious pest of maize in North America and in Europe. The larvae feed upon maize roots and weaken the plants, making them prone to lodging and difficult to harvest. Annual rotation between maize and soybean crops has been recognized as an effective strategy for controlling *D. v. virgifera* for almost a century (Levine et al. 2002). The technique exploits the female's ovipositional fidelity to maize and the univoltine lifecycle in which eggs overwinter in the soil. Under rotation, eggs laid into maize crops hatch into soybean crops, the roots of which the larvae cannot consume. At the same time, rotated maize crops remain free of larvae because soybean was grown the previous year and was not an attractive crop for oviposition. Unfortunately, the effectiveness of crop rotation has been severely compromised during the last two decades. Damage to first-year (i.e. rotated with soybean) maize was first seen in Ford County, Illinois in 1987 (Levine and Oloumi-Sadeghi 1996). The problem has subsequently expanded to cover most of Illinois as well as parts of a number of neighbouring states (O'Neal et al. 2002).

The rapid expansion outward from an initial focus suggests that selection has favoured a variant form of *D. v. virgifera* that is able to circumvent crop rotation. Studies of populations in the field have shown that a change in *D. v. virgifera* behaviour is associated with the failure of crop rotation. Elevated densities of adult *D. v. virgifera* are found in soybean fields in areas where crop rotation has become ineffective (Spencer et al. 1997, 1998, 1999b). More importantly, increased rates of oviposition outside maize fields are also observed in these areas (Spencer et al. 1997; Rondon and Gray 2004). Variant populations do not appear to be attracted to soybeans (Spencer et al. 1999a) but do exhibit significantly greater locomotive activity (Knolhoff et al. 2006) and may therefore be more prone to leave and oviposit outside maize fields. Computer simulation studies have shown that selection for an allele causing reduced ovipositional fidelity at a single locus is sufficient to explain the observed phenomenon (Onstad et al. 2001). However, to date no such locus has been identified.

Although a difference in locomotive activity has been observed between variant and wild-type *D. v. virgifera*, it is not sufficiently pronounced to provide an individual diagnostic behavioural assay (Knolhoff et al. 2006). In the absence of a behavioural test, a molecular marker for variant *D. v. virgifera* is desirable. Such a marker would allow the distribution of variant *D. v. virgifera* in North America to be better characterized and an evaluation of its presence or absence in Europe to be made. Unfortunately, such a molecular marker has proved somewhat elusive to date. Studies of microsatellite variation have not detected any general genetic differentiation between variant and wild-type *D. v. virgifera* (Miller et al. 2006), in keeping with the general lack of population-genetic structuring throughout the US corn belt (Kim and Sappington 2005a). It therefore seems probable that potential molecular markers linked to the loss of ovipositional fidelity will be confined to regions of the genome linked to the gene or genes responsible for the variant behaviour.

The demographic history (i.e. population sizes and migration patterns) of a population is common to all loci in the genome. However, a particular selective factor will affect only the variability of the gene(s) under selection and, to an extent, loci linked to them. Consequently, diversifying selection, which favours different alleles in different populations, will tend to increase the degree of allele frequency divergence between populations compared with the neutral case. Conversely, stabilizing selection, which favours the same allele in different populations, will tend to reduce the degree of allele frequency divergence. Thus, if allele frequency divergence is measured at multiple loci, those under selection may be detected as outliers compared with the distribution of allele frequency divergence expected under neutrality (Lewontin and Krakauer 1973). It can be assumed that diversifying selection is responsible for the variant behaviour of *D. v. virgifera* because alleles responsible for the variant will be favoured where crop rotation is common but disadvantageous where crop rotation is rare because variants will waste a proportion of their eggs ovipositing away from continuous maize crops. Obtaining the distribution of allelic frequency divergence expected under the neutral hypothesis analytically is not straightforward, because migration produces correlations in allele frequencies among demes (see Beaumont 2005 for a review). However, with modern computational tools, the neutral distribution can be estimated by simulation and is generally robust to factors such as correlated allele frequencies (Vitalis et al. 2001; Beaumont et al. 2002).

The advent of a variety of techniques that allow populations to be typed for large numbers of molecular markers has resulted in a renewed interest in scanning genomes for evidence of natural selection (Beaumont 2005). One of the most accessible methods of high-throughput genotyping for non-model species is amplified fragment-length polymorphism (AFLP) (Vos et al. 1995). AFLP markers apparently linked to genome regions subject to natural selection have been identified in several studies covering a variety of animal taxa and potential selective agents (Wilding et al. 2001; Campbell and Bernatchez 2004; Bonin et al. 2006). In the study reported here, we performed a genome scan using AFLP to search for genetic markers potentially linked to genes under selection that enable the *D. v. virgifera* variant to circumvent crop rotation.

2 Materials and Methods

2.1 Sample collection

Three samples of *D. v. virgifera* were collected during late June and early July 2005 from three sites in central Iowa, approximately 200 km west of the western limit of the known distribution of the variant phenotype. We refer to these as 'wild-type' samples below. The sample sites were Curtiss Farm, Ames (42°00.308′N; 93° 40.187′W), Johnson Farm, Ames (41°58.913′N; 93°38.849′W) and Ankeny (41°43.055′N; 93°36.316′W). Samples of *D. v. virgifera* were also collected near Champaign-Urbana, east-central Illinois, where the variant phenotype has been present for at least 10 years (Levine et al. 2002). These samples are referred to as 'variant' samples below. The problem of variant western corn rootworm in this region has resulted in the widespread adoption of both soil insecticide treatments and transgenic maize that expresses *Bacillus thuringiensis* (*Bt*) toxin in the roots. Consequently, sampling for variant western corn rootworm was confined to three untreated experimental plots, separated by approximately 500 m at 40°09.291′N; 88°08.770′W. The distance between the Iowa and Illinois sampling sites was approximately 500 km. Between 50 and 100 individuals were collected per sample.

The wild-type samples were collected from continuous (i.e. at least second-year) maize crops whereas the variant samples were collected from first-year maize following soybean. Individuals were sampled at the pupal stage. *D. v. virgifera* larvae are unable to disperse over distances much in excess of 1 m (Hibbard et al. 2003, 2004). This ensured that the individuals were sampled from the same field into which they had hatched. Thus, individuals of the variant samples were known to be the offspring of variant females that had oviposited in soybean crops. In addition, the sampling of pupae minimized the risk that contaminating maize DNA in the gut would be detected during AFLP genotyping. Although the sampling strategy targeted the variant vs. wild-type distinction, the two sample types were not sympatric. Thus, for any loci that appear to be influenced by selection the selective agent could either be crop rotation or some other environmental difference such as patterns of insecticide usage or climate. Any loci identified as under selection should therefore be considered candidates for an association with the variant behaviour that should be verified by further investigation.

Western corn rootworm pupae cannot be easily distinguished by morphology from those of northern corn rootworm, *Diabrotica barberi* in the field. It was therefore necessary to determine each individual's species identity on the basis of its microsatellite genotype. To minimize the risk of over-sampling siblings, only one pupa was collected from the root system of a given plant. Pupae were located by digging up lodged, wilted or unusually small maize plants and searching the soil surrounding the root system. The collected pupae were placed into 95% ethanol in the field and transferred to 50-ml screw-cap centrifuge tubes packed with cotton wool saturated with 95% ethanol for shipping to the INRA laboratories at Sophia-Antipolis, France. Upon arrival, the pupae were transferred to fresh tubes of 95% ethanol and stored at −20°C.

2.2 DNA extraction and microsatellite genotyping

Prior to DNA extraction, the pupae were washed at least three times in 0.065% NaCl solution to remove both ethanol from the tissues and soil particles from the cuticle. Each pupa was then placed in a 1.5-ml microcentrifuge tube, frozen under liquid nitrogen and pulverized using a microcentrifuge tube pestle. DNA was extracted from the pulverized pupae using DNEasy tissue kits (Qiagen, Hilden, Germany) following the manufacturer's protocol, including an RNase treatment step. DNA was recovered in two separate elutions of 200 μl from the DNEasy kit spin columns.

The second, less concentrated elution was used as template for each of two multiplex polymerase chain reaction (PCR) reactions amplifying a total of eight microsatellite markers. The first reaction amplified loci DVV-D2, DVV-D4, DVV-D11 and DVV-T2 (Kim and Sappington 2005b) and the second amplified loci DVV-D5, DVV-D8, DVV-D9 and DVV-ET1 (Kim and Sappington 2005b; Miller et al. 2005). Each multiplex PCR was carried out in total volume of 10 μl containing 2 μl of template DNA solution, 1X Qiagen Multiplex PCR mix and each primer at 200 nM. Forward PCR primers were 5′ labelled with a fluorescent dye to allow the PCR products to be detected on an automated DNA sequencer. To equalize the signal strength between markers, labelled primers were combined with unlabelled forward primers for several loci in the following proportions (labelled:unlabelled): DVV-D2 3 : 1; DVV-D4 3 : 1; DVV-T2 3 : 1; DVV-D5 3 : 1; DVV-D9 1 : 15; DVV-ET1 3 : 1. The thermal cycling program for both multiplex PCRs was 95°C for 15 min followed by 25 cycles of 94°C for 30 s, 55°C for 90 s and 72°C for 60 s followed by a final incubation at 60°C for 30 min. Amplified microsatellites were analysed by electrophoresis in combination with GeneScan-500 LIZ size standards (Applied Biosystems, Foster City, CA) on an ABI PRISM 3100 automated DNA sequencer (Applied Biosystems). The calculation of the sizes of amplified microsatellites and their assignation to allele classes was partially automated using the GeneMarker computer program, version 1.40 (SoftGenetics, State College, PA). Determination of each individual's microsatellite genotype allowed the species to which it belonged to be discerned. Of the microsatellites tested, only DVV-D9, DVV-T2 and DVV-ET1 can be amplified from*D. barberi* whereas all loci can be amplified from *D. v. virgifera* (Kim and Sappington 2005b). Individuals from which additional loci were amplified could therefore be classified as *D. v. virgifera*.

2.3 AFLP genotyping

The concentration of the first (most concentrated) DNA elution was measured by UV absorbance at 260 nm for all individuals positively identified as *D. v. virgifera*. Digestion of genomic DNA with the restriction enzymes *Eco*RI and *Pst*I, ligation of adaptors and PCR pre-amplification was carried out as described by Hawthorne (2001) except that 500 ng genomic DNA was used as starting material rather than 2 μg. To reduce the risk of misleading results because of contaminating DNA, all steps in the AFLP protocol up to the preparation of the pre-amplification PCRs were conducted under a cell-culture hood. Prior to digestion with restriction enzymes, each individual was assigned at random to a position in a 96-well PCR plate. This position was retained throughout the AFLP genotyping process (i.e. restriction, adaptor ligation, pre-amplification and selective amplification). Each 96-well plate also included at least one negative control without DNA.

Selective AFLP amplifications were conducted using primers with two selective bases each. The *Eco*-selective primers were 5′ end-labelled with the fluorescent dye 6-FAM to allow detection on an automated DNA sequencer. Prior to genotyping all the sampled individuals, selective primers were tested on a panel of four individuals to identify primer combinations that gave acceptable numbers of easily scored amplicons. All possible combinations of the following *Eco* + 2 and *Pst* + 2 primers were evaluated (nomenclature as specified by Keygene NV, Wageningen, the Netherlands): E11 (+AA), E13 (+AG), E17 (+CG), E21 (+GG), E24 (+TC), P13 (+AG), P16 (+CC), P21 (+GG) and P24 (+TC). The following primer combinations were selected to genotype the full set of samples: E13 + P16, E13 + P24, E17 + P13, E17 + P21, E17 + P24, E21 + P21, E21 + P24, E24 + P21, E24 + P24. Polymerase chain reaction conditions for the selective amplifications were as described by Hawthorne (2001). The amplified fragments were separated by capillary electrophoresis on an ABI Prism 3100 DNA sequencer along with GeneScan-500 LIZ size standards. Fragment sizes were estimated by comparison with the size standards using the GeneMarker software. GeneMarker was also used to classify fragments into allelic bins and to record their presence or absence in each individual. Each AFLP fragment was identified by a code in the form E–P^n, where 'E' is the identifier of the *Eco* selective primer, 'P' that of the *Pst*-selective primer and n the estimated size of the fragment in base pairs.

2.4 Data analysis

All analyses of the microsatellite data were performed using the Genepop version 3.4 computer package (Raymond and Rousset 1995). Fisher's exact tests on tables of genotypic counts were used to test each sample for conformance to Hardy–Weinberg genotypic proportions and on tables of allele counts to test the homogeneity of allele frequencies among samples. Fisher's method (Sokal and Rohlf 1995) was used to combine P-values over all loci. The degree of allele frequency heterogeneity among samples was quantified by estimating F_{ST}, the proportion of the total genetic variation because of differences between samples (Weir and Cockerham 1984).

The R computer program (R Development Core Team 2006) was used to perform exact tests on tables of AFLP fragment counts to test for homogeneity among samples and Fisher's method was used to combine P-values over loci. The Ddatacal program, which is distributed with Dfdist (Beaumont and Balding 2004) was used to estimate F_{ST} at AFLP loci, based on Zhivotovsky's (1999) Bayesian estimator of allele frequency for dominant loci. Dfdist was then used to test for loci with F_{ST} values greater or lower than expected under selective neutrality (Beaumont and Nichols 1996). Briefly, this method takes a trimmed mean F_{ST} as an estimator of the average F_{ST} for selectively neutral loci. Simulations are then performed using the coalescent and an island model of migration to generate the distribution of F_{ST} conditional on heterozygosity for the given neutral average. The observed data are then compared with this distribution and loci that fall outside specified confidence limits can be identified as having significantly elevated or reduced F_{ST} values. For both Ddatacal and Dfdist, the parameters $a = 0.25$ and $b = 0.25$ were used for the beta-distributed prior of the Bayesian allele frequency estimator. The trimmed mean F_{ST} was calculated by discarding the upper and lower 30% of F_{ST} values. Fifty thousand simulated loci were generated using Dfdist with the parameter $4N\mu$ set to 0.6 and the target-neutral F_{ST} determined as described in the Results

section below. Observed F_{ST} values, conditioned on heterozygosity, were considered to be significantly greater or less than neutral expectations if they fell outside the upper or lower 2.5% of the simulated distribution (i.e. a two-tailed test at the 5% significance level). This analysis was performed on the full set of samples and on each pairwise combination of samples. Although the pairwise analyses are less powerful, they are useful for demonstrating that any outlier loci show excess divergence in comparisons between wild-type and variant samples but not in comparisons made within each type of sample. The robustness of the results to variations in the simulation parameters was evaluated as follows: The effect of varying $4N\mu$ was evaluated by repeating the analysis of the full set of samples with $4N\mu = 0.2$ and 1.0. The effect of varying the parameters of the beta prior was evaluated by repeating the pairwise analyses with $a = 0.5$ and $b = 1.0$. Finally, the effect of modifying the target F_{ST} was evaluated by repeating the analyses of all samples, Illinois and Iowa with a target F_{ST} of 0.001.

3 Results

3.1 Microsatellite variation

No *D. barberi* were detected in the samples from Illinois. *D. barberi* were present in the samples from Iowa and were particularly abundant at the Curtiss farm site. Of the Iowa individuals for which a microsatellite genotype was successfully obtained, 8.3%, 77.7% and 5.4% were *D. barberi* at the Ankeny, Curtiss Farm and Johnson Farm sites, respectively. The numbers of confirmed *D. v. virgifera* at Ankeny, Curtiss Farm and Johnson Farm were 44, 27 and 52, respectively. The numbers of confirmed *D. v. virgifera* from the three Illinois samples were 44, 47 and 46, respectively. The final sample sizes were as large or larger than several previous genome-scanning studies that have successfully identified loci that are affected by natural selection (Vitalis et al. 2001; Campbell and Bernatchez 2004; Emelianov et al. 2004; Bonin et al. 2006).

All eight microsatellite loci were polymorphic with between three and 21 alleles per locus. No significant deviations from Hardy–Weinberg genotypic proportions were detected at any locus in any sample. Exact tests on tables of allele frequencies revealed significant heterogeneity between the samples (table 1). The heterogeneity was due to differences between Iowa and Illinois as there was no significant overall heterogeneity among samples from the same state (table 1). The degree of heterogeneity among the samples, as measured by the estimate of F_{ST}, was 0.005 (table 1). Thus, the microsatellite loci indicated a weak but significant genetic differentiation between the two states.

3.2 AFLP variation

A total of 365 AFLP fragments were scored. Of these, 253 were considered polymorphic with the estimated frequency of the more common allele being $\leqslant 0.98$. In keeping with the results for microsatellite loci, there was evidence for an overall heterogeneity among the samples in the observed number of fragments when individual P-values were combined by Fisher's method ($\chi^2 = 578.86$, d.f. = 506, P = 0.0134). This heterogeneity was also due to differences between the Illinois and Iowa samples as there was no significant heterogeneity between samples from the same state. The average estimated F_{ST} over all 253 loci was 0.002 and the trimmed mean, excluding the upper and lower 30% of values was estimated as −0.001.

Table 1. *P-values for allele frequency homogeneity exact tests and estimates of F_{ST} per microsatellite locus, over all loci, per state and over all samples*

	All		Iowa		Illinois	
	P-value	F_{ST}	P-value	F_{ST}	P-value	F_{ST}
DVV-D2	0.0075	0.0087	0.9345	−0.0054	0.8706	−0.0054
DVV-D4	0.5042	−0.0011	0.2921	0.0046	0.5262	−0.0056
DVV-D5	0.7842	−0.0034	1.0000	−0.0132	0.2500	0.0081
DVV-D8	0.4298	0.0052	0.8755	−0.0012	0.1965	0.0099
DVV-D9	0.0613	0.0051	0.0801	0.0097	0.3007	−0.0040
DVV-D11	0.0334	0.0050	0.1965	0.0068	0.6349	−0.0032
DVV-T2	0.0301	0.0152	0.0230	0.0259	0.2562	0.0112
DVV-ET1	0.3455	0.0036	0.0933	0.0214	0.8776	−0.0096
All	0.0096	0.0053	0.1020	0.0066	0.6073	−0.0006

The slightly negative estimated trimmed mean F_{ST} suggested that the appropriate average neutral F_{ST} for the analysis using Dfdist should be zero. However, under the coalescent simulation model implemented by Dfdist, this results in an infinite immigration rate and all events in the genealogy being migrations rather than coalescences so that the simulation would never reach a common ancestor (M. A. Beaumont, personal communication). Consequently a small, positive target F_{ST} of 0.005 was used. This rendered the analysis somewhat conservative for detecting loci with greater than expected F_{ST} such as those associated with the difference between variant and wild-type *D. v. virgifera*. Conversely, the analysis was non-conservative for detecting loci with lower than expected F_{ST} because of stabilizing selection.

The Dfdist analysis of all the samples together led to the detection of three loci with significantly elevated global F_{ST} values: E17-P21^{434}, E21-P24^{242} and E24-P21^{106} (fig. 1). Exact tests revealed significant overall heterogeneity in the presence of the fragment among samples for all three loci, P-values being 0.0026, 0.0069 and 0.0001 for E17-P21^{434}, E21-P24^{242} and E24-P21^{106}, respectively. When samples from Iowa or Illinois were considered separately, locus E17-P21^{434} did not exhibit significant heterogeneity within either state. However, locus E21-P24^{242} exhibited significant heterogeneity among samples from Iowa (P = 0.0232), as did locus E24-P21^{106} among samples from Illinois (P = 0.0041). Thus, locus E17-P21^{434} was the only one that showed both a significantly elevated F_{ST} and a consistent differentiation between variant *D. v. virgifera* and their wild-type counterparts. This was due to a generally higher frequency of the amplified fragment in the variant samples from Illinois (fig. 2). These results agreed with those of the pairwise Dfdist analysis in which E17-P21^{434} was the only locus that

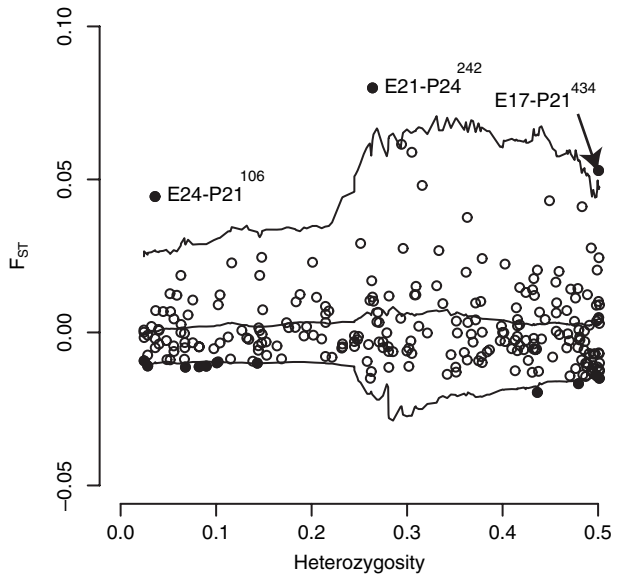

Fig. 1. *Plot of global F_{ST} vs. heterozygosity for 253 AFLP markers. Solid lines indicate the mean and 95% confidence intervals for selectively neutral loci as determined by simulation. Filled circles indicate loci falling outside the confidence limits. Loci with significantly elevated F_{ST} are labelled with their identifying code*

showed significant excess divergence in wild-type vs. variant comparisons but never for comparisons within each of the two groups of samples (fig. 3). The rather weak divergence at E17-P21^{434} was also apparent in the pairwise analysis as the locus exhibited significant excess divergence in only five of the nine wild-type vs. variant comparisons (fig. 3). These results were robust to variations in the simulation parameters. Varying $4N\mu$, a and b did not change the results at all. Reducing the target value of F_{ST} did result in more loci showing significant excess heterogeneity. However, locus E17-P21^{434} remained the only locus that showed excess heterogeneity when all samples were compared but not within Illinois and Iowa.

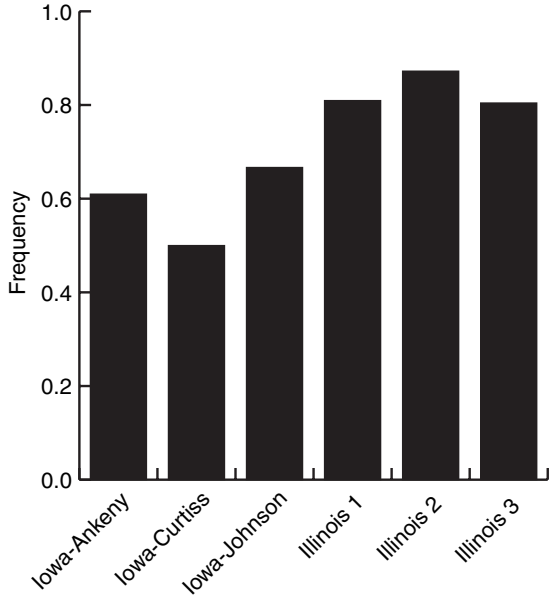

Fig. 2. *Frequencies of the amplified fragment at locus E17-P21^{434} in each of the samples*

4 Discussion

This study showed weak but significant general genetic differentiation between samples from Illinois and those from Iowa at both microsatellite and AFLP loci. This result contrasts with those of previous studies of *D. v. virgifera* population-genetic structure in the US corn belt. A study that included samples from throughout the corn belt and several other locations in the US did not find any significant genetic heterogeneity among the samples with the exception of comparisons between Texas vs. other locations and Illinois vs. Pennsylvania and Delaware (Kim and Sappington 2005a). In particular, there was no evidence of heterogeneity between Illinois and Iowa. A second study, targeted towards the crop-rotation variant examined multiple samples from Illinois including samples both from within and outside the variant problem area also failed to detect any significant differentiation from the Iowa data of Kim and Sappington (2005a) at the same microsatellite loci that were used in the present study (Miller et al. 2006).

It is possible that the discrepancy between this study and the earlier ones is simply due to a weak geographical population structuring that was not detected by the earlier studies. An alternative possibility is that there is a weak genetic differentiation between variant and wild-type *D. v. virgifera* arising from a reduced level of gene flow between the two (i.e. a degree of assortative mating). This study would probably have greater power to detect such differentiation because pupae were sampled whereas previous studies have sampled adults. Assuming that the variant has not entirely replaced wild-type beetles in east-central Illinois, samples of adults from this region are likely to contain a mixture of wild-type and variant individuals because of adult dispersal after emergence. The presence of wild-type adults in the east-central Illinois sample would tend to obscure any differentiation from, for example, samples of pure wild type from Iowa. Furthermore, if the differentiation between wild-type and variant *D. v. virgifera* is weak, the reduction in observed heterozygosity (Wahlund effect) expected after the two types have mixed via adult movement between fields might also go undetected. It is interesting to note that although allele frequencies in the Illinois sample studied by Kim and Sappington (2005a) were not significantly different at the 5% level from those of other corn belt samples, and principal coordinate analysis suggested that Illinois did not group with the rest of the corn belt (see Fig. 4 in Kim and Sappington 2005a). Although the results of this study do not demonstrate that a general genetic differentiation between wild-type and variant *D. v. virgifera* exits, they do re-open the question as to whether one does. Any future study directed towards this question will require a carefully planned and comprehensive sampling strategy.

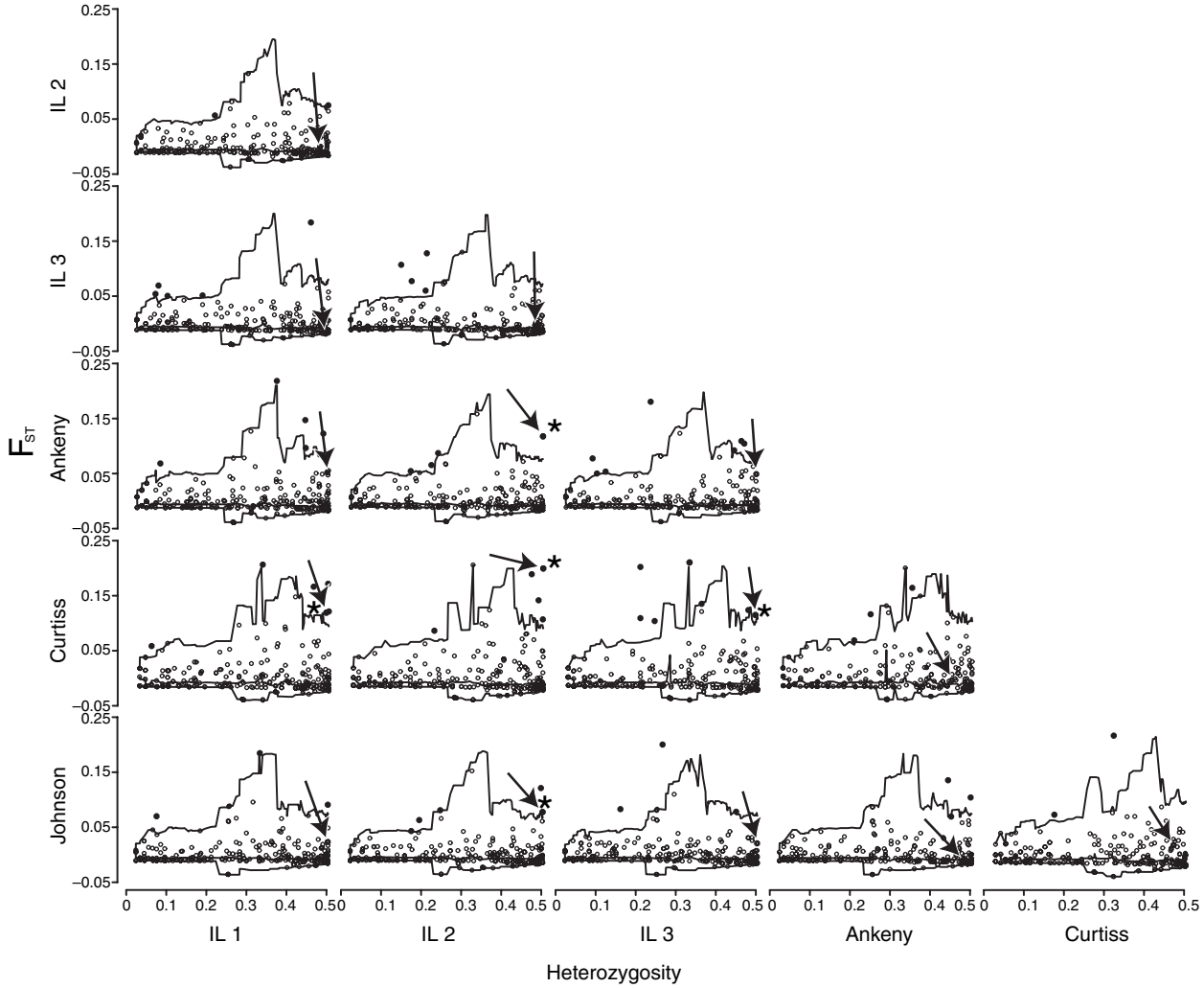

Fig. 3. *Plots of F_{ST} vs. herozygosity for each pairwise combination of samples. Locus E17-P21^{434}, the only one to show significant excess divergence between wild-type and variant samples but never between samples of the same type, is indicated with an arrow. Cases where the observed F_{ST} at locus E17-P21^{434} exceeds the 95% confidence limit determined by simulation are marked with an asterisk*

The AFLP-based genome scanning element of this study identified a single AFLP locus, E17-P21^{434}, that could be considered as a candidate for association with the variant–wild-type distinction. This locus exhibited both a significantly elevated global F_{ST} and homogeneity within the two groups. However, although the frequencies of the amplified fragment were significantly different between the Iowa and Illinois samples, it was reasonably common in both sets of samples (fig. 2) and certainly not a diagnostic marker. Given the number of AFLP markers examined and the 5% significance level used, it is possible that the detection of this locus is due to chance rather than a genuine association with a gene involved in determining variant or wild-type behaviour. If E17-P21^{434} truly is linked to such a gene, the linkage is probably loose given the similar frequencies of the amplified fragment in the Iowa and Illinois samples.

The fact that markers more strongly associated with the variant–wild-type distinction remained undetected may be due to a number of reasons. First, the *D. v. virgifera* genome is rather large at approximately 2.5 Gbp (Sappington et al. 2006). This study employed 253 polymorphic AFLP fragments giving an average of ca. 1 marker per 10 Mbp. Given this low density of marker coverage, there is a low probability that any of the markers would be linked to a gene involved in the determination of the variant behaviour. This is especially true if the number of genes with a major effect on the variant behaviour is low, as suggested by the model of Onstad et al. (2001). This can be remedied by the addition of more markers, which is technically straightforward now that the basic protocols for performing AFLP genotyping of *D. v. virgifera* have been established. However, the economic feasibility of adding enough markers to significantly improve the chances of detecting genes of interest will need to be considered. Secondly, all of the AFLP markers used were generated using the same pair of restriction enzymes, *Eco*RI and *Pst*I. If recognition sites for either of these enzymes are not randomly distributed throughout the genome, the location of the AFLP marker will also be clustered (Young et al. 1999). If the gene or genes of interest are located in genome regions

where *Eco*RI/*Pst*I AFLP fragments are rare, the chance of detecting the genes will be further reduced. This risk could be ameliorated by genotyping the samples for AFLPs derived from other restriction enzymes. Finally, an implicit assumption of the genome scanning approach applied here is that the variant behaviour is controlled by one or a few genes of major effect. If the probability of ovipositing away from maize is a quantitative trait controlled by many genes, each of small effect, the chances of identifying a diagnostic marker will be small, even with a high density of marker coverage. These concerns notwithstanding, the generally low level of genetic differentiation between variant and wild-type populations is encouraging. A marker linked to a gene with a substantial influence on ovipositional behaviour should be readily apparent by genome scanning against the low background F_{ST}.

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